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Introgressive Hybridization between Native and Invasive Crayfish: A Test of Reproductive Isolation

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INTROGRESSIVE HYBRIDIZATION BETWEEN NATIVE AND INVASIVE CRAYFISH: A TEST OF REPRODUCTIVE ISOLATION

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Abstract

The objective of this project was to investigate the evolutionary relationship and ecological interactions between the two crayfish populations: *O. virilis* and *O. quinebaugensis*. These two populations were compared genetically by examining the COI mitochondrial gene and a microsatellite-containing nuclear locus, and morphologically by measuring the chelae and curvature of the male gonopods. Mating trials were used to determine whether there are reproductive barriers between the populations other than geographic isolation. The results of this project suggested that these two crayfish populations are in the process of speciation. However, since some interhaplotype mating trials were successful, introgressive hybridization may occur in nature where the crayfish cohabitate.

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Introduction

The crayfish species *Orconectes virilis* is hypothesized to be invasive species in the New England area (USGS.gov), which is also home to a crayfish of a related lineage which is hypothesized to be native: *Orconectes quinebaugensis* (Mathews and Warren, 2008). While the evolutionary and ecological relationship of these two crayfish populations was not previously well understood, it is known that invasive species can sometimes have detrimental effects on related native species. Invasive species can directly or indirectly interact with closely related native species, which can lead to possible competition for food, habitat and reproduction (Wilson et al., 2008). These interactions can lead to the displacement of the native species, and have even been proposed as a mechanism for the extinction of native species (Mooney and Cleland, 2001). Risk of extinction is even greater if an invasive and native species hybridize and introgress, which has been shown to occur in other *Orconectes* crayfish (Perry et al., 2001a, b; Perry et al., 2002).

It is therefore necessary to develop an understanding of the evolutionary relationship and ecological interactions between the *O. virilis* and *O. quinebaugensis* populations. The purpose of this project was to investigate the reproductive interactions of the two populations in the laboratory to determine whether the *O. quinebaugensis* population is at risk due to the introduction of *O. virilis.* In order to understand this investigation, it is necessary to understand what is meant by species, the concepts of invasive species and hybridization, and the previous crayfish studies related to this project.

Definition of Species

There are several different ways to define a species in biology. This presents problems when trying to categorize organism into different species. One of the definitions states that two organisms are separate species if they cannot breed with one another (Mayr, 1957). This definition suggests that hybridization between two species cannot occur and if they do the two parent lineages would not be defined as species. Therefore, reproductive isolation is not always an adequate definition of a species (Mayr, 1996). Another definition suggests that different species are capable of interbreeding, but their offspring will be weaker and most likely infertile

because of the genetic differences between species (Mayr, 1996). In some cases, the hybrids are better adapted than the parental species (Stevens, 1974).

Depending on which definition is followed, there can be conflicting conclusions drawn from the same data set. For example, based on the first definition, if any offspring are produced by the mixture of the two organisms they are the same species. The second definition states that hybridization can occur but the offspring will be weaker or infertile. The second definition also suggests that the chance of individuals from two distinct species hybridizing is less than the chance of two individuals of the same species breeding.

Most studies rely on the phylogeny of the species in order to distinguish between two species. Rees et al. (2002) used this method in analyzing the difference between *Hyalomma dromedarii, Hyalomma truncatum,* and *Hyalomma marginatum rufipe.* The variances in the populations were distinguished through phylogenetic analyses of the COI gene. McPeek and Wellborn (1998) analyzed the differences between amphipod populations. This study relied on genotype frequencies to quantify the structures of the populations. The study started with the different phenotypes and used the genetic data to confirm the differences between the species. Inversions in genes have also been used to determine the differences between the populations and categorize the species such as in Noor et al. (2001), which examines the difference in certain *Drosophila.* All of these studies based their discussions on the phylogenetic data that was discovered rather than the typical theoretical discussions. Since there are many definitions for species, it may be more practical to investigate the evolutionary and ecological relationships between the two different populations rather than categorize them.

Invasive Species

An invasive species is defined as a group of organisms which has been artificially introduced to and become established in an area beyond its natural range (Keller et al., 2008). Any type of living organism can theoretically become an invasive species, including plants, animals, fungi and microbes. New species can be introduced into a new habitat through a variety of routes, and can be introduced on a relatively small range such as transport from one watershed to another, or on a large range such as relocation between continents (Mooney and Cleland, 2001). Human interference, both intended and unintended, often leads to the introduction of invasive species (Keller et al., 2008).

The intricate transportation networks created by humans have a great effect on the introduction of invasive species. Many non-indigenous species are introduced into new areas because of their accidental transport across biogeographical barriers, and a small fraction of these introduced species become established and therefore invasive (Mooney and Cleland, 2001). Relocations of organisms can also inadvertently occur because of loss of habitat due to land-use alteration and changes in biogeographical barriers. An example of this type of change in barrier is the construction of canals which creates a link between previously isolated bodies of water (Mooney and Cleland, 2001). These introductions of new species can ultimately lead to a change in the ecosystem of the affected area.

Not all invasive species cause problems within an ecosystem, but some introductions may result in a negative impact. The introduction of invasive species can have a variety of consequences for native species as well as the nonindigenous species. This introduction can cause competition between the invasive and native species, leading to a possible displacement or extinction of certain species (Mooney and Cleland, 2001). If a species is introduced into an area where another related species already exists, the possibility of displacement is even greater, especially if there is competition over food, habitat and reproduction (Wilson et al., 2008).

In North America, introduction of invasive crayfish has become increasingly common. Crayfish introductions have been mostly attributed to their use as bait, food and aquarium pets, since they are sometimes released into an area which is not native to them (Wilson et al., 2004). Non-native crayfish can also be introduced if human land use, such as changes to natural and artificial dams and the addition of canals, leads to the removal of geographic barriers between crayfish populations (Lodge et al., 2000). Past studies suggest that these introductions have caused changes to ecosystems. Introduction of invasive crayfish has lead to the displacement of native crayfish species, and changes of biodiversity within a habitat (Wilson et al., 2004; Hill et al., 1999). According to the Global Invasive Species Database (www.invasivespecies.net), the crayfish species *O. virilis* has a wide native range in North America, but it is also hypothesized as invasive in many areas including the Northeast (Figure 1: Distribution of O. virilis, Credit: USGSFigure 1), where *O. quinebaugensis* is located (Mathews and Warren, 2008).

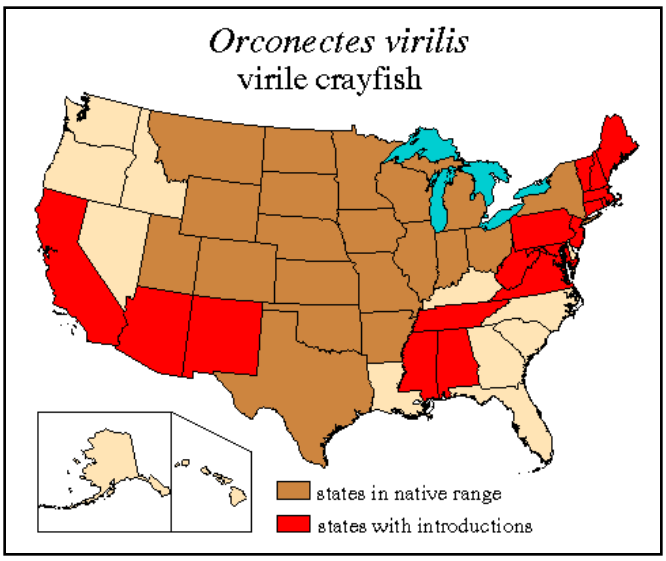

Figure 1: Distribution of O. virilis, Credit: USGS

Hybridization between Invasive and Native Species

A hybrid is the offspring of genetically different parents (Purves et al., 1995). From this base definition, any living organism whose parents have different genotypes is a hybrid, but from a larger standpoint, the term hybrid refers to the offspring of two organisms whose genetic differences may be due to their being members of different subspecies, species, genera, or families. As we use the term species, hybrids may occur, but may have reduced fitness.

Hybrids in nature are more often formed between plants than animals, and the offspring of such hybrid plants are more likely to be fertile than animal hybrids. While the species barrier is more preserved in animals due to complex mating behaviors in addition to other pre- and post-zygotic barriers, in plants these factors are more often geographic location and flowering times. In addition, many plant hybrids acquire a trait known as hybrid vigor or heterozygote advantage, in which the offspring from two different species fares better than either of its parents in natural selection (Stevens, 1974).

While hybridization can create fertile or sterile hybrids, there is also at least one case in the animal kingdom of a hybrid becoming its own species. Known as the Lonicera fly, it is the hybrid of the blueberry maggot (*Rhagoletis mendax*) and the snowberry maggot (*R. zephyria*), two species native to the United States. This fly feeds on honeysuckle (*Lonicera* spp.) a plant which is not native to North America but is an invasive species from Asia introduced 250 years ago. This illustrates an important concept for hybrid biology: if the hybrid can fill a distinct

niche, it can evolve as a species separate from its parent species, as happens commonly in the plant kingdom (Schwarz et al., 2005). Other members of the animal kingdom, however, may not produce a separate species as sterile hybrids may occur.

Ecologically, there are potential problems that can arise from hybridization. For example, introgressive hybridization can be problematic. Introgression occurs when a fertile hybrid offspring breeds with a member of one or both of its parent species and introduces genes into the parental populations (Lapedes, 1974). Introgressive hybridization can be detrimental when a smaller population interbreeds with a larger one. If fertile hybrids are produced in the first generation, they can interbreed with either of the parent species. This can threaten the genetic integrity of the smaller population: as the hybrids continue to interbreed with the smaller original population, genes from the other population are introduced slowly until the majority of the population contains those genes (Perry et al., 2002).

Introgressive hybridization of an invasive species with a native one could cause the extinction of the native species or a loss in fitness (Mooney et al., 2001). Mallard ducks, for example, have hybridized with, and reduced the non-hybrid populations of, the New Zealand gray duck, the Hawaiian duck, and the Florida mottled duck, largely through introgression. This has resulted in populations of ducks that show more and more traits of mallard ducks (Rhymer and Simberloff, 1996). The Sitka deer from Japan was introduced to Great Britain over a century ago, and have hybridized with the native red deer population. This is apparently threatening the genetic integrity of the red deer in some areas (Abernathy, 1994).

However, there is also the possibility that a hybrid will be able to fill a niche and contribute to the local ecosystem. Besides the Lonicera fly described earlier, *Spartina alterniflora*, a form of grass that inhabits coastal areas, was introduced to Britain during the early 1800s. It hybridized with the local species *S. maritima*, which produced a sterile hybrid. Then, through a process known as chromosome doubling, the hybrid became fertile and created the aggressive species *S. anglica*. This new species is able to occupy tidal flats that were unavailable to either of its parent species, and both its parent species survive in Britain in limited distributions (Anttila et al, 1998). Thus, hybrids could be either beneficial or harmful to the environment depending on the conditions, and hybrids of invasive and local species are no exception. A beneficial hybrid may have a stabilizing effect on the environment by filling a niche, while a harmful hybrid could upset the balance of the ecosystem and destabilize it, for

instance by hunting key organisms necessary to the survival of many other organisms in the environment.

When there are negative genetic consequences associated with hybrids between two species or populations, these species may evolve isolating barriers that prevent or reduce this hybridization (Hardig et al., 2000). This process occurs by reinforcement, in which natural selection leads to the evolution of isolating barriers between populations. If two species hybridize, and the offspring have lower fitness than their parent species, then there is no evolutionary adaptation for those species to hybridize. Over time, the individuals which avoid hybridization through pre-zygotic barriers, and therefore have a higher fitness, will begin to outnumber those with lower fitness. This will result in the evolution of reproductive barriers between the populations (Epifanio and Philipp, 2001). This may include both prezygotic and postzygotic barriers, although prezygotic barriers are more likely to occur than postzygotic barriers because if the organism is prevented from mating in the first place, then no resources are wasted forming a zygote that will not survive to pass on its genes. If the pre-zygotic barriers are strong enough, then the species may be considered reproductively isolated: where members of one species do not hybridize with members of the other species. Reinforcement may play an important role in the later stages of speciation as it can lower the occurrence of hybridization and the possibility of introgression (Hoskin et al., 2005).

Phylogeography and Patterns of Invasion in Orconectes

There are over 350 species throughout North America, with at least six known species in Massachusetts (EPA, 2009; Fetzner, 2008). The genus *Orconectes* is local to eastern North America, ranging from Canada to Mexico. There are more than 80 different taxa of *Orconectes* in North America, which comprise approximately 21% of all the crayfish species on the continent. Even though North America is a center for crayfish biodiversity, the evolutionary history of this group is not well understood.

A previous study attempted to better understand this phylogreography of the *Orconectes* genus by collecting crayfish which were believed to be part of the *O. virilis* complex from different sites, including sites in New England. These crayfish were studied to understand their genetic and morphological relationship (Mathews et al., 2008). In this study, six different mitochondrial lineages of the *O. virilis* complex, including the crayfish species *O. nais* and *O.*

deanae, were identified. Along with finding differences in the mitochondrial DNA (mtDNA) among these six mitochondrial lineages, some morphological differences were also found. Specifically, a difference was found in the length and curvature of the central projection of the sexually mature (form I) male gonopod (Figure 2) (Mathews et al., 2008). One of the six lineages identified was later described as *O. quinebaugensis*, and this group differed significantly in gonopod morphology from other crayfish of the northeast *O. virilis* complex species (Mathews and Warren, 2008). Both morphological and genetic data were therefore used in this project to further investigate the relationship between *O. virilis* and *O. quinebaugensis*.

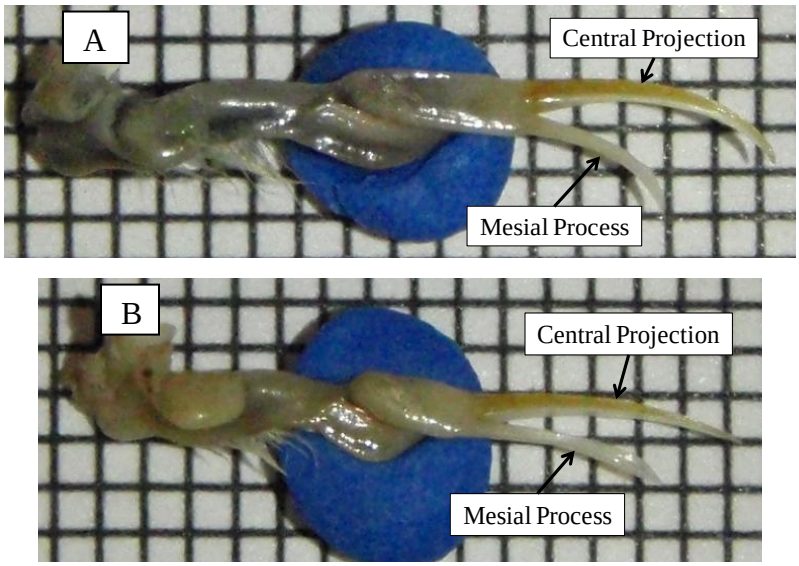

Figure 2: First male gonopods of individuals from two collection sites used in this investigation (a) M2 and (b) R1. See Methods for further details about collection sites.

Other studies have been interested in the occurrence of invasions by *Orconectes* crayfish and how these invasions affect native populations and ecosystems. Perry et al (2002) described interactions among *Orconectes* taxa, including an invasion by the rusty crayfish, *O. rusticus*, and its interactions with the native *O. virilis* and *O. propinquus*. Included in this review were explanations as to why *O. rusticus* was taking over aquatic ecologies and displacing the native crayfish species. *O. rusticus* was found to grow faster and reach a greater body size than *O. propinquus* (DiDonato and Lodge, 1993; Hill et al., 1993; Garvey et al., 1994), and to have larger chelae, a greater growth rate, and a higher rate of survivorship than *O. virilis* (Lodge et al., 1986; Garvey and Stein, 1993; Hill and Lodge, 1999). In addition, there was a possibility that *O rusticus* was displacing, or extirpating, *O. propinquus* via hybridization and introgression. Morphology was used to study this possibility of introgression (Crocker and Barr, 1968; Capelli

and Capelli, 1980; Smith, 1981; Berrill, 1985; Butler and Stein, 1985). However, there were significant morphological differences between *O. rusticus* populations that made morphology an inefficient mode for detecting hybridization (Capelli and Capelli, 1980; Tierney, 1982).

In a later paper, diagnostic nuclear and mitochondrial markers were used to determine that hybridization occurred between *O. rusticus* and *O. propinquus*, but not with *O. virilis* (Perry et al, 2001a, b). In addition, the fitness of hybrid offspring and competitive ability of adult hybrids was studied. There was no significant difference seen between the fitness of hybrids or intraspecfic offspring. However, it was seen that hybrid adults were competitively superior to both parental species. This study also indicated that the majority of the F1 hybrids were the offspring of *O. rusticus* females and *O. propinquus* males, and that said offspring were more likely to backcross with *O. rusticus* than *O. propinquus*. This study shows that introgression is possible between these two species, however, it is unclear what consequences will arise due to introgression in these populations, whether the alleles of *O. propinquus* will survive in the *O. rusticus* or whether those alleles will be selected against.

Objectives and Rationale

As previously described, *O. virilis* has a wide natural range across North America, but is hypothesized to be invasive in some additional areas including New England (USGS.gov). *O. quinebaugensis* is a recently discovered crayfish population which is closely related to *O. virilis* (Mathews and Warren, 2008; Mathews et al., 2008). Due to its recent discovery, there is not very much information about its distribution although it has been found in various locations throughout Massachusetts, in some cases coexistent with *O. virilis*. It is currently hypothesized that *O. quinebaugensis* is native to New England, although there is too little evidence to draw conclusions at this point. *O. virilis* has two sister taxa in addition to *O. quinebaugensis*: *O. deanae* and *O. nais* (Taylor and Knouft, 2006). *O. deanae* was first described as having a small range in New Mexico but has recently been discovered in Oklahoma (Bergey et al., 2004). *O. nais* has a range throughout the mid-west including Oklahoma and Kansas (Smith, 1979).

Although *O. virilis* is often hypothesized as invasive in New England, its actual origin in the area is relatively unknown. Crocker (1979) described the distribution of *O. virilis* in New England as a patchwork, but suggested that this may be caused by either natural distribution or human introductions. However, the study referenced known transfers of *O. virilis* between lakes

in different states in the 1960s. In addition, the study also described *O. virilis* as a popular food which was often transferred to New York from the mid-west, explaining that introduction of *O. virilis* into New England could have been possible. Although it is possible that *O. virilis* is invasive in New England, it is difficult to draw conclusions without further evidence.

The goal of this project was to examine the reproductive interactions of *O. virilis* and *O. quinebaugensis* populations in the lab. In addition to testing the reproductive ability of the crayfish, the populations were compared morphologically and investigated genetically using sequences of the mitochondrial gene (cytochrome oxidase I) and a microsatellite-containing nuclear locus. This provided us with an understanding of the ecological interactions and evolutionary relationship of the populations. This information can be used to determine whether introgressive hybridization is possible and whether either is at risk of extinction due to this relationship.

Methods

Crayfish Collection and Care

Crayfish were collected from two sites, the Quinebaug River in Sturbridge, MA (M2) and the Blackstone Gorge in North Smithfield, RI (R1). These sites were chosen from the results obtained by Mathews et al. (2008), in which two mitochondrial clades were found in New England. These sites were each found to contain representatives of only one of the two clades: *O. virilis* (R1) and a lineage later described as *O. quinebaugensis* (M2) (Mathews and Warren, 2008). In addition, morphological differences were observed between the male gonopods of the crayfish at each of the sites (Buckholt and Mathews, personal communication).

Crayfish were collected by hand and net at both sites. At least 45 females and 45 form I (sexually mature) males and were collected at each site for this project, and any extras were later euthanized by freezing. Males that molted to form II (non-breeding) after collection were removed from the studies. The target crayfish were distinguished from other species by the coloration typical of the *O. virilis* complex. Collection took place in early September to collect virgin female crayfish, as very few mating pairs had been seen at the sites. These crayfish were transported back to WPI with males and females in separate buckets to avoid possible mating.

Crayfish collected at each site were always kept separately from those of the other site and the back of each crayfish was labeled with their site and a unique number.

All crayfish were stored in separate tanks with flower pots for shelter and air stones. These tanks were located in rooms with day-night light cycles which were adjusted weekly to emulate the natural outdoor cycle. The water temperature of the tanks was also adjusted as needed to mirror the outdoor temperatures, down to 10°C as this was the minimum for the system. Crayfish were fed three mornings a week until December, then twice a week from December to March as all crayfish needed less food due to the drop in temperature. Crayfish were alternately fed shrimp pellets and broccoli. On feeding days, crayfish were inspected for any deaths or molts. Male crayfish were kept in tanks with a filtering system. These tanks were siphoned clean approximately once a month, when excess food particles were present. Female crayfish were kept in separate tanks without a filter. These tanks were siphoned and refilled three afternoons a week, after feeding. After breeding experiments were complete, males were euthanized by freezing and the filter system previously containing the males was emptied and cleaned and the females were moved to these tanks.

DNA Extraction

DNA was extracted from every crayfish using Gentra Puregene reagents and the protocol described for tissue in the Gentra Puregene handbook. Tissue was extracted from the second leg behind the claw on the left side of each crayfish and placed directly into an Eppendorf tube containing 300 μL of cell lysis solution. This solution caused the tissue cells to burst, and the content of the cells to be released. To these tubes, 1.5 μL of Puregene proteinase K was added to break down the proteins in the cells, and the mixture was incubated overnight at 55° C to ensure complete lysis. After incubation, the mixture was cooled in an ice bath and 100 μL of protein precipitation solution was added to the tubes and vortexed at high speeds for 20 seconds. Each tube was centrifuged at 16000 x g for three minutes. This caused protein to precipitate out of the solution and form a pellet, leaving the DNA and other materials in the supernatant. The supernatant was then transferred to a new Eppendorf tube containing 300 μL of isopropanol. This tube was gently inverted 50 times to mix the solutions and then centrifuged for one minute at 16000 x g. This caused the DNA to precipitate out of the solution and form a pellet. The supernatant was discarded and the tube was inverted and drained on a paper towel, while taking

care not to dislodge the pellet. The pellet was then washed with 300 μL of 70% ethanol, inverted several times and centrifuged for one minute at 16000 x g for one minute. Once again, the DNA formed a pellet and left any remaining materials in the supernatant. The supernatant was discarded and the tube was drained on a paper towel, then the pellet was air dried for 15 minutes. Once dried, 50 μL of DNA hydration solution was added to the tube to dissolve the DNA in the solution and the sample was incubated for 1 hour at 65° C. Once this process was complete, the sample was stored in a freezer for later use.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method used to amplify the amount of a specific piece of DNA containing a targeted gene. This method for amplifying DNA was first described by Kary Mullis (Mullis et al., 1986). The components of PCR are the DNA containing the gene of interest, a forward and a reverse primer which are complimentary to the DNA which flanks the gene of interest, a DNA polymerase, deoxynucleoside triphosphates (dNTPs), magnesium chloride ($MgCl_2$) and a buffer. DNA polymerases are enzymes which replicate single stranded DNA by adding complementary base pairs to create a second strand. In PCR, the polymerases begin replicating from each of the primers. Since replication can only occur in the 5' to 3' direction, the primers must also be 5' to 3'. The $MgCl_2$ provides magnesium ions, which help in the binding of the primers and serve as a cofactor in DNA polymerization. The dNTPs provide a mixture of nucleotides, which are used as needed to create the second strand. (Voet et al., 2008)

PCR takes place in a thermal cycler and occurs in several different steps. The sample is first heated to around 95° C to denature the double stranded DNA into two separate strands. The sample is then cooled to an annealing temperature appropriate for the primers being used, during which the primers hybridize to the complimentary DNA. Lastly, the sample is heated to around 72°C for the polymerase to extend complimentary strands of DNA starting at each primer. Once this step is complete, the cycle is repeated as many times as necessary. Every cycle doubles the amount of targeted DNA. Since the denaturing step of PCR reaches such high temperatures, it is necessary to use a polymerase which can withstand this heat. Most DNA polymerases would inactivate at this temperature, meaning that new polymerases would need to be added after each cycle. To avoid this, Taq polymerase, which is isolated from the theromophilic bacteria *Thermus aquaticus*, is used as it can withstand high these high temperatures. (Gilbert, 2006)

For this project, the gene of interest was cytochrome c oxidase I (COI), a mitochondrial gene which encodes for a protein in complex IV of the mitochondrial electron transport chain (Voet et al., 2008). This gene was of interest in this study, and is commonly used in other phylogenetic studies for several main reasons. First of all, as a mitochondrial gene, COI is haploid as it is only passed to an offspring from its mother and therefore doesn't undergo recombination (Hebert et al., 2003). This is useful because it means that an organism's mitochondrial DNA has only one ancestral line which doesn't change apart from mutations (Avise, 2004). Second, evolution rate is higher in mitochondrial DNA than nuclear DNA. This is due partly to higher rates of mutation in mitochondrial DNA which occurs because of inefficient repair mechanisms. In addition, the coalescence time, or the time since the two haplotypes diverged, is shorter in mitochondrial DNA because of it can quickly drift within a population, causing differences within closely related populations (Beebee and Rowe, 2008). Since COI is a protein-coding gene, substitutions occur even more commonly. This exceptionally high rate of mutation allows COI to be used to distinguish divergences between different species as well as reproductively isolated groups within a species.

The primers used to amplify the COI gene were Orco-COIF3 [5'-AATGTGGTAGTTA-CAGCTC-3'] and Orco-COIR2 [5'-GCCTCTTTTTTACCAGACTC-3']. Each PCR sample was set-up with a final volume of 20μL, with the following components: 0.8μM of each primer, 0.16mM dNTP, 1x of Taq polymerase buffer, 0.2 units of Taq polymerase, 2.5mM of $MgCl_2$, 1μL of extracted DNA (diluted to 1:10), and water to bring the volume to 20μL. The PCR was initially run for 2 minutes at 95°C. Then each cycle ran as follows: 30s at the denaturing temperature of 95°C, 30s at the annealing temperature (50°C for COI) and 60s at the extension temperature of 72°C. This PCR was run for 40 cycles and ended with a final extension at 72°C for 10 minutes to ensure that all DNA was polymerized. The thermal cycler then dropped to 4°C until the sample was stored in a refrigerator.

Gel Electrophoresis

Gel electrophoresis was used to determine whether the PCR was successful in this project. Gel electrophoresis is a common method used to separate DNA fragments by size. DNA fragments are negatively charged at biological pH levels because they are made up of charged nucleic acids. Electrophoresis uses this characteristic to separate molecules by running a current

through an agarose gel through which the DNA can travel. DNA fragments run towards the positive end of the gel, while the agarose gel creates a matrix through which the DNA fragments must travel. Small fragments are able to travel through the gel easier and therefore travel faster through the gel than larger fragments. To use gels to determine the approximate sizes of DNA fragments, a marker, containing fragments of known sizes, is run along with the samples on the same gel. The distances traveled by these fragments can then be compared to the distance traveled by the DNA sample (Voet et al., 2008).

The expected DNA fragments from the PCR performed were approximately 550 base pairs long. They were therefore run on a 2% NuSieve Agarose gel, which was appropriate for separating fragments of this size. This gel was prepared in 1x TBE buffer by adding powdered agarose to the buffer and heating until the solution appeared clear. Once cooled slightly, the gel was poured into a mold and cooled until it was solidified. A solution of loading dye and Sybr green was created (1:10) and 2 μL of this loading buffer was mixed with 2 μL of PCR sample and loaded into the gel. Hyperladder V was also mixed with Sybr green (1:10) and 4 μL of this mixture was loaded into one well for each row of wells. Gels were run at 120 V until the blue loading dye had traveled approximately ½ the way through the gel. The gel was then photographed under UV light so that the DNA samples were visible.

DNA Sequencing

After the gene of interest was amplified using PCR, the reaction products were cleaned using exonuclease I - shrimp alkaline phosphatase (exoSAP) protocol which uses exonuclease to remove excess primer and SAP to remove excess dNTPs (Nucleics, 2009). Then the product was sequenced using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit, which uses the chain-terminator method first developed by Sanger (1977). This kit sequences DNA in a process similar to PCR, but only uses one primer at a time. The components of each sequencing reaction were 1x of Big Dye buffer, 0.30 μL of Big Dye 3.1 terminator mix, 1 μL of 3.3 μM of one of the primers, 8.5μL of water and 1.5 μL of PCR product. The mixture was run in a thermal cycler for 40 of the following cycles: 96°C for 5 seconds, 50°C for 5 seconds and 60°C for 4 minutes, after the 40 cycles were complete, the cycler was run at 15°C for 15 minutes, and 4°C until removed. The Big Dye terminator mix contains a polymerase, a high concentration of dNTPs and a small concentration of fluorescently labeled dideoxynucleoside triphosphates (ddNTPs). These

ddNTPs are essential to the sequencing process, as they provide a mixture of base pairs which lack the 3' OH group, which is essential in binding to the next nucleotide. Due to this missing group, whenever these ddNTPs are incorporated into a chain, the chain terminates with that nucleotide. By using only a small concentration of ddNTPs, DNA strands of every possible length, each differing by one base pair, are created. Capillary electrophoresis is used to separate the different strands by size, and different colored fluorescent labels are used to determine the sequence of the DNA (Voet et al., 2008). The facility where the samples were sent for sequencing ran the products on the 3730xl Genetic Analyzer and processed the data utilizing Peak trace software by Nucleics in order to process the samples

DNA Analysis

Once the DNA samples were sequenced, the results needed to be analyzed. First, the Seqman module of the DNA analysis software Lasergene was used to align and confirm the forward and reverse sequences. Once this was done, all samples were aligned using Bioedit (Hall, 1999) or the Megalign module of Lasergene. Once aligned, the sequences were observed at several diagnostic base pairs (Mathews & Warren, 2008), to determine which mitochondrial haplotype sequence represented.

Crayfish Mating Trials

Mating seasons of *O. virilis* and *O. quinebaugensis* are in the autumn, females tend to lay their eggs between the months of March and May (Mathews, personal communication). Male crayfish have two different forms, between which they switch by molting. Form II males are the non-breeding form and cannot mate, while form I males are sexually mature. In form I males, the morphology of the first gonopod are modified into a reproductive form. This change is visible and can be used to distinguish between the two forms (Figure 3). While form shows whether males are ready to reproduce, females have a different indicator. The readiness of females to reproduce is indicated by the white glair glands. The glair glands are formed after the annual molt in August. They are visible as white tissue found ventrolaterally on the abdominal somites (Reynolds, 2002). Mating trials were therefore performed once females presented with developed glair glands. Mating and egg-laying in the laboratory has been previously successful. One trial performed in the laboratory had an average mating success rate of 48%. Out of the

successfully mated females, 59% of them laid eggs, while only 30% of the successfully mated females produced fertile eggs (Warren, Personal Communications).

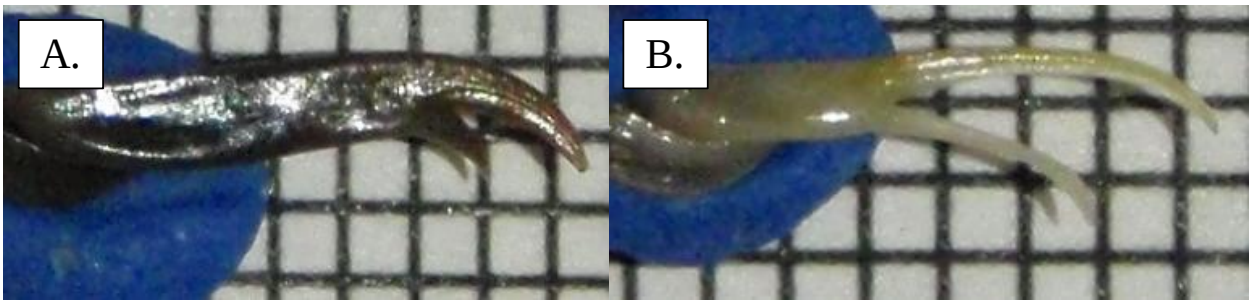

Figure 3: Tip of male pleopod from (a) a form II male and (b) a form I male.

For our mating trials, crayfish were placed into four treatment groups: R1 female x R1 male; R1 female x M2 male; M2 female x R1 male and M2 female x M2 male. A total of 24 trials in each treatment were carried out, but several were excluded due to the detection of form II males. Once each crayfish was placed in a treatment group, the carapace length was measured for both males and females. This is the length from the tip of the rostrum to the top of the abdomen (Figure 4). Crayfish were then matched up for mating by their carapace length, by sorting the males and females and matching them by ascending size. Mating experiments were performed in individual tanks for three consecutive hours. Tanks were rinsed and refilled between each individual mating to get rid of any chemical signals that were present in the water. Except for mating, crayfish were kept separate leading up to the experiment to avoid any chemical signaling.

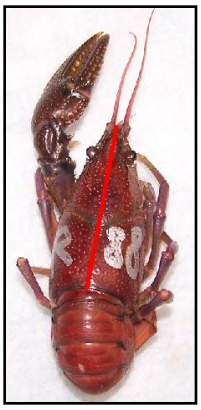

Figure 4: R1-88 with line showing measured length of carapace

Each mating experiment was digitally recorded by a hard-drive camera, and the footage was uploaded onto disks for analysis. Each camera was set-up to record 4 mating tanks to

maximize video quality. All mating experiments took place between the hours of 8 am and 4 pm because mating pairs are frequently seen in the field during daylight hours (Buckholt and Mathews, personal communication). After mating, female crayfish were returned to their original tanks, and males were euthanized by freezing. Males were later transported into ethanol for preservation. Once all mating experiments were complete, each video was viewed and the observer recorded whether crayfish attempted to mate and whether they were successful. To avoid bias in observations of mating trials, labels were removed from disks in attempts to blind the viewer. Mating attempts were defined as any attempt to get into mating position and mating was considered successful when the crayfish appeared to be in mating position and remained relatively still (Figure 5). Any ambiguous results were scored by another viewer to reach a consensus.

Figure 5: Example of crayfish in the mating position. (Warren, 2008)

Following the mating experiments, females were observed twice a week to see if they had laid any eggs. The females were kept on a similar feeding and light cycle as previously described with minor adjustments to compensate for what the natural environment would be like. The light cycle became shorter as the winter months progressed and then increased again as spring came again. The temperature decreased as the outside weather became cooler and increased again in the warmer months. Observers recorded the date the eggs were first discovered and whether the eggs developed or rotted.

Measurements

We took measurements of two morphological characteristics that appeared to differ between the two populations. First, we measured the length and width of the chela. The width of the chela was measured across the widest part of the palm. The length was measured from the base to the tip of the immovable finger (Figure 6). The left chela of the crayfish was measured unless the crayfish was missing its left chela. The measurements the chelae were performed on the crayfish using calipers.

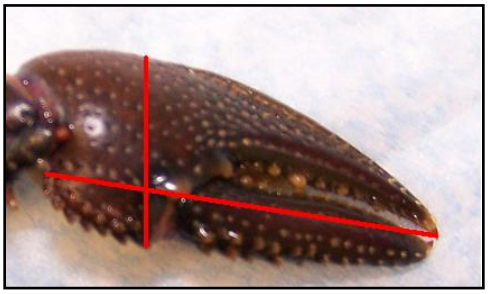

Figure 6: Chela of R1-88 with lines showing measured length and width.

Second, we measured the angle of curvature of the central projection and the mesial process of the first gonopod in all Form I males. The left gonopod was removed from male crayfish and photographed on 1-mm grid paper. The gonopods were held in place by clay. The photos were taken using the "super macro LED" setting of the camera. With the program TpsDig2 (Rohlf, 2005), the lengths of the upper and lower projections were measured. The length of was measured from the split between the upper and lower projections to the tip of each part of the gonopod. In order to determine the angle, the halfway points of these lengths were found. A new point was then created to measure the angle. The new point was at the top of each projection at a distance (from the split) equal to the previously measured halfway point. The angle was then measured by using the split point, the halfway point at the top of the projection, and the end of the gonopod (Figure 7). Both the angles of the upper and lower projections were measured and recorded.

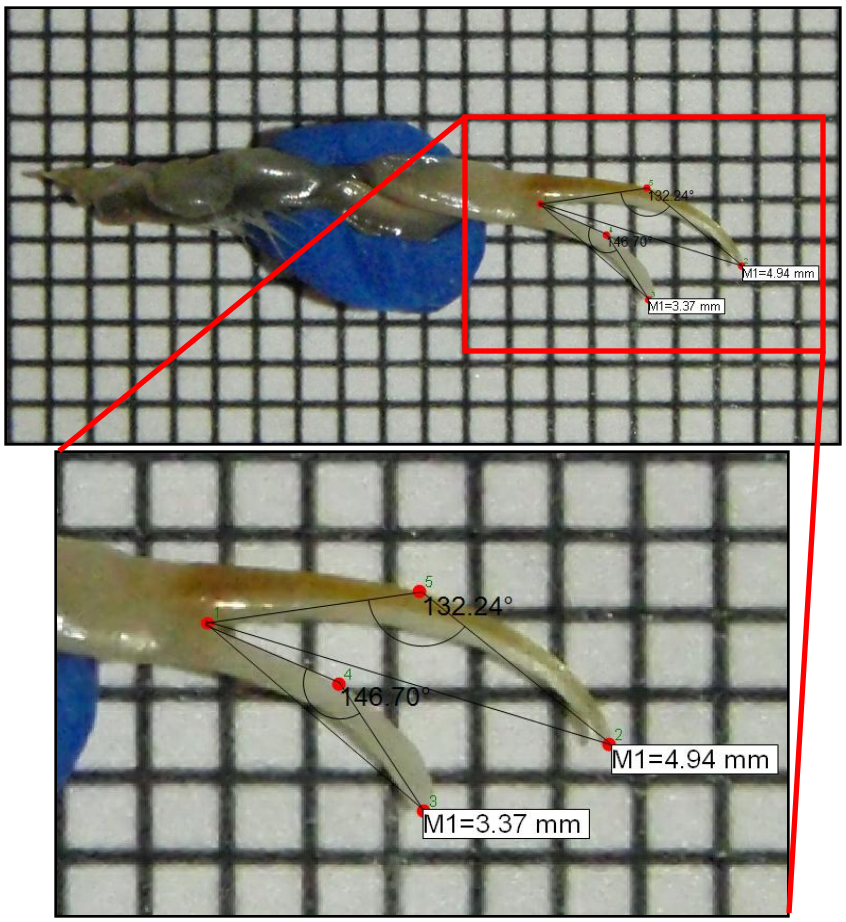

Figure 7: Sample measurements of M2-54 gonopod, showing measurement points for length and angle of both upper and lower projections.

Data Analysis

To determine whether the results of this project were significant, most data were statistically analyzed. The morphological measurements of each population were compared using histograms in Microsoft Excel, and the means of these data were calculated. To determine whether these means were significantly different between the two populations, independent sample t-tests were performed using SPSS (version 14.0). Using this test, a p value of under 0.05 suggests that the means are statistically different. To determine whether there was a correlation between carapace length and other morphological measurements, linear regression was performed using SPSS and the coefficients of determination (R^2) were used to determine if there was a relationship between the two characteristics. The R^2 is a value between 0 and 1, where 1 indicates a complete correlation between the x and y value while 0 indicates there is no correlation at all.

For the mating trial data, Pearson's chi-square tests of independence were performed using SPSS (version 14.0) to determine whether the results of these trials were significant. This test was used to determine whether the number of mated crayfish was statistically different than those who did not mate in each treatment. The null hypothesis in this case was that there would be the same amount of crayfish that mated and did not mate. In a Pearson's chi square test, the null hypothesis can be rejected if the p value is lower than 0.05.

A logistic regression analysis was performed using SPSS (version 14.0). This type of analysis is used to determine whether a certain set of continuous and/or categorical characteristics (predictors) can be used to predict a categorical outcome. In this project, logistic regression was used to indirectly determine whether the two populations of crayfish were genetically and morphologically diverged enough to be distinguishable from one another. This was tested by examining whether gonopod curvature, chela ratio and genotype could be used as predictors for what mitochondrial haplotype each male crayfish possessed. This was not tested for female since there would only be two predictors in the model. A logistic regression analysis works by creating a prediction model using the variables or characteristics as predictors. The analysis gives a percent of how well the model works with the data presented, and each predictor is given a p value for how significant they are to the model. A p value of 0.05 or lower can be used to reject the null hypothesis that the predictor is not significant to the model.

Results

Population Genetics

For a total of 176 crayfish (site M2: N=88; site R1: N=88), DNA samples were extracted and the COI mitochondrial gene was sequenced. These sequences were compared and diagnostic nucleotides were used to determine what mitochondrial haplotype each crayfish had. In addition, allele size data were provided for a nuclear microsatellite locus. All crayfish from the R1 site shared the haplotype consistent with *O. virilis* (Mathews et al., 2008). At the M2 site 85% of the crayfish shared the haplotype considered to represent *O. quinebaugensis* (Mathews and Warren, 2008) while all others shared the *O. virilis* haplotype (Table 1). A strong difference was seen in the genotype distribution at each site (Table 1): 87% of the M2 population had the BB genotype while 76% of the R1 population had the AA genotype. Each site also contained a number of AB

individuals. We also noticed a strong relationship between microsatellite genotype and mtCOI haplotype (Figure 8) with the majority of individuals with the Oq haplotype sharing the BB genotype, and the majority of those with the Ov haplotype sharing the AA genotype.

Table 1: Occurrences of mitochondrial COI haplotypes at sites M2 and R1

	mtCOI Haplotype		Genotype		
	Oq	Ov	AA	BB	AB
Individuals from M2 Site	74	13	1	76	10
Individuals from R1 Site	0	88	67	8	13

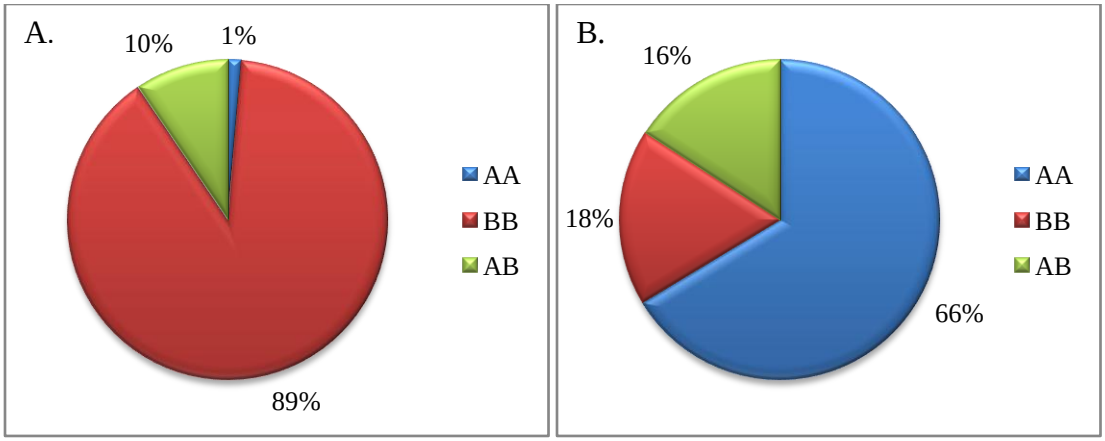

Figure 8: Distribution of genotypes for haplotypes (a) Oq and (b) Ov

Morphological Investigation

The carapace length of each crayfish was measured and the means of each population were found (Figure 9). The R1 population had a significantly smaller mean carapace length than the M2 population (t_{82}=4.807, p<0.001).

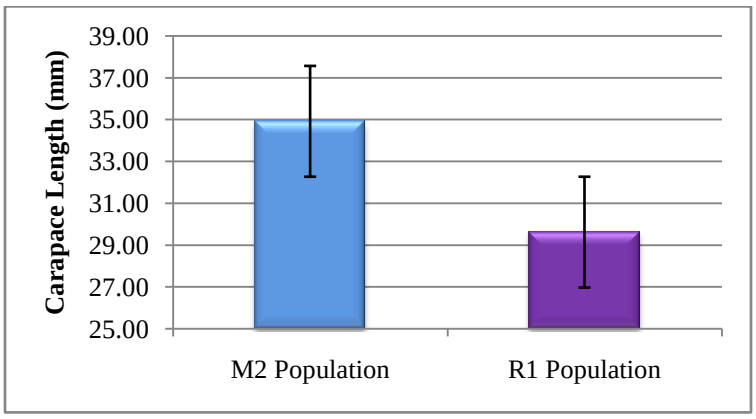

Figure 9: Mean carapace length of crayfish with standard error bars

For all form I male crayfish, the angle of curvature of the central projection and the mesial process of the gonopods were measured. These data were compared between the M2 and R1 population. According to the means, both curvature angles are smaller for the M2 population (Figure 10). The two populations had significantly different curvature angles for both the central projection (t_{82}=-18.78, p<0.001) and the mesial process (t_{82}=-10.53, p<0.001). To determine whether there was a correlation between the angles of the central projection and mesial process curvature angles, a linear regression was performed and an R^2 value was calculated. The R^2 was 0.6, indicating a moderate correlation. Due to this correlation, only the central projection curvature angle was used in the logistic regression.

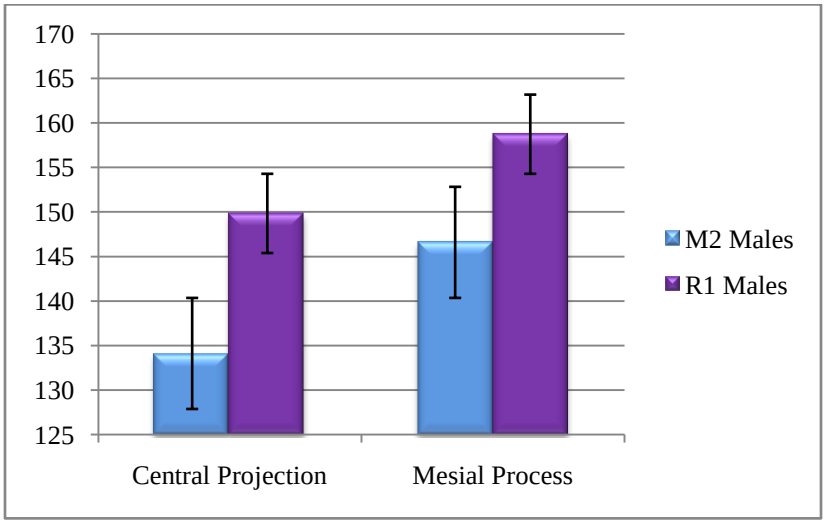

Figure 10: Mean curvature of central projection and mesial process of male gonopods with standard error bars

The chelae width and length was also measured for each crayfish, however, data from discovered form II males was excluded from the analysis. To determine if there was a difference in chelae shape between the R1 and M2 populations, a ratio of width to length was calculated for each sample (Figure 11). An independent sample t-test showed that the chelae ratios of the two populations were significantly different (t_{162}=-7.27, p<0.001).

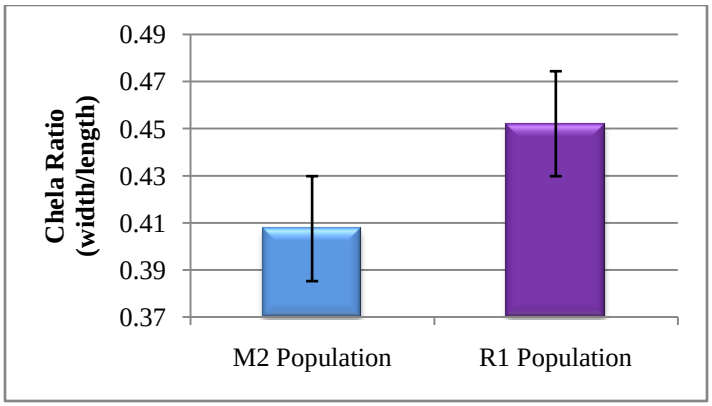

Figure 11: Mean chelae width/height ratio with standard error bars

To determine whether there was a correlation between carapace length and each of the other morphological measurements, linear regression was used and an R^2 value was calculated for each comparison. There was a small correlation between the carapace length and the central projection (R^2=0.18) and between the carapace length and the mesial process (R^2=0.32). The correlation between the carapace length and the chelae ratio was even smaller (R^2=0.07). Since these correlations were so small, this means that the three morphological measurements used to compare the two populations were mostly independent of crayfish body size.

Logistic Regression

A logistic regression was used to determine if there was any relationship between the morphological data, the genotype and the mitochondrial haplotype. A model containing the genotype, central projection curvature of the gonopod and the chela ratio could be used to correctly predict the mitochondrial COI haplotype with an accuracy rate of 90.2% for the data collected in this project (Table 2). It was found that genotype, central projection curvature and chela ratio were all statistically significant to the model (Table 3: $p<0.05$).

Table 2: Success of haplotype classification model with all predictors

Observed		Predicted		
		Haplotype		Percent Correct
		Ov	Oq	
Haplotype	Ov	43	6	87.8
	Oq	2	31	93.9
Overall Percentage				90.2

Table 3: Statistical significance of each variable as predictors in the logistic regression model for haplotype

	Wald	df	p
Central Projection Curvature	4.917	1	0.027
Chela Ratio	4.508	1	0.034
Genotype	4.395	1	0.036

Mating Trials

Crayfish were divided by site into four treatment groups and paired for mating. For each of these treatment groups, the percent of mating attempts and percent of successful mating trials were calculated (Figure 12; mating pairs are depicted as female x male). The percent of attempts and success were also totaled for intrapopulation trials and interpopulation trials. There were significantly more successful intrapopulation (N=26) than interpopulation (N=10) mating trials (χ^2 =12.23, p<0.001), however, we noted that R1 x R1 pairs were more successful than M2 x M2 pairs.

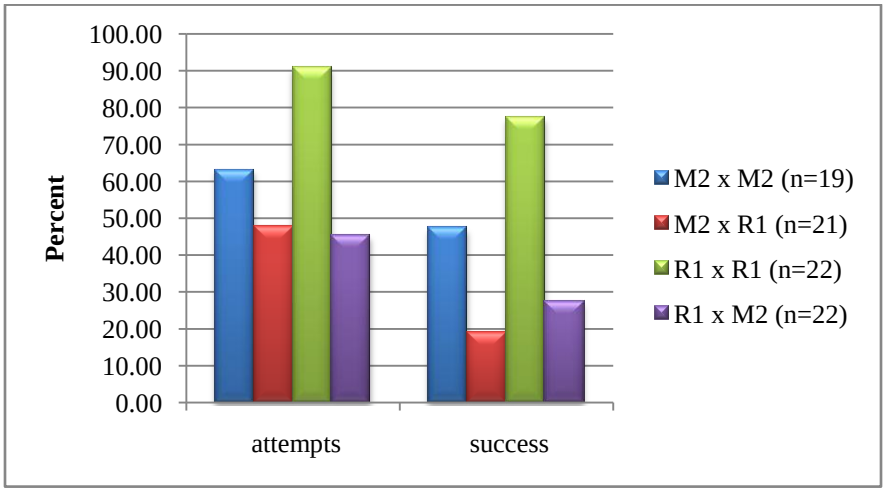

Figure 12: Results of mating trials by site (n represents the number of trials in each treatment group)

Since we found that the M2 site contained a small number of crayfish which shared the mitochondrial haplotype consistent with *O. virilis*, the mating trials were also examined by this haplotype (Figure 13). These data also showed that there were significantly more intrahaplotype mating trials than interhaplotype mating trials (χ^2 =14.23, p<0.001) and Ov x Ov pairs were also more successful than Oq x Oq pairs.

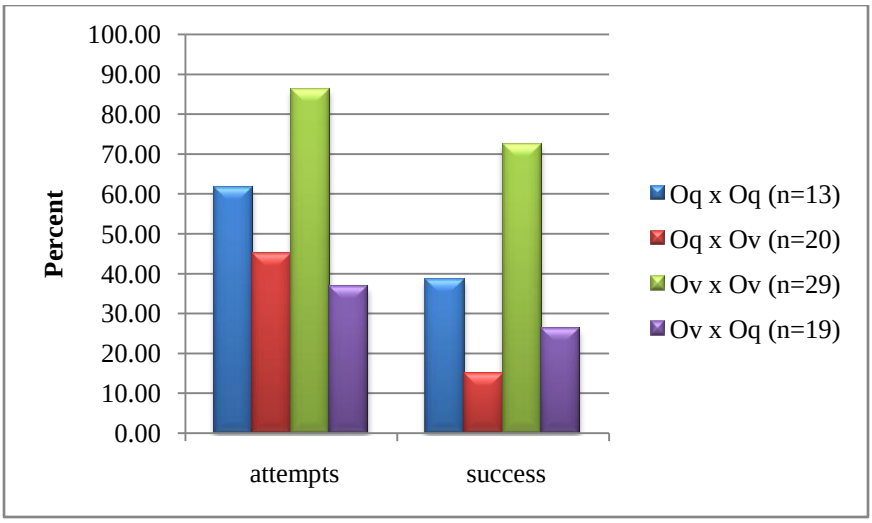

Figure 13: Results of mating trials by site (n represents the number of trials in each treatment group)

As we found there was a difference in the mean carapace length of each population, we investigated whether this affected the mating success. Since the interpopulation mating trials had a wider range of size differences, only these trials were considered in the correlation of size difference and mating attempts and success. The mating success was highest when pairs were closely size matched, and lowest when there was a large size difference (Figure 14). However, sample sizes at the extremely different size pairs were small.

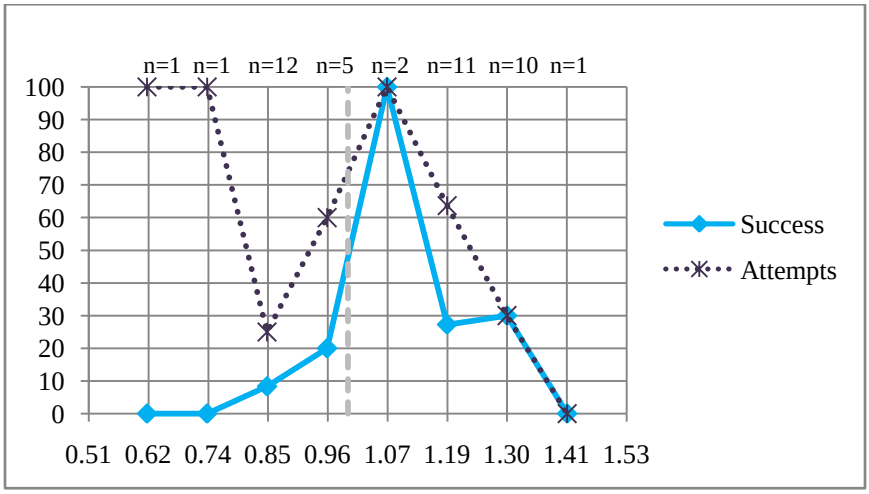

Figure 14: Percent of interpopulation mating attempts and mating successes (n represents the number of mating pairs in each size group). The dotted grey line represents where males and females are perfectly size matched. Everything to the right line represents males larger than females, and everything to the left represents females larger than males.

After females were mated, they were monitored for the presence of eggs twice a week. We also monitored whether the eggs developed or rotted. Developing eggs occurred in both mated females and non-mated females although there were more in mated females. The presence

of eggs was compared for females which were part of intrahaplotype mating trials and those that were part of interhaplotype mating trials (Figure 15). Intrahaplotype pairs resulted in more females with eggs than interhaplotype pairs. However, since some non-mated females had developing eggs, these females may have mated in the field before collection which makes it difficult to draw conclusions from the data. There is a large discrepancy seen between the number of females with eggs and the number with developing eggs because some eggs had clearly rotted while others had not yet developed and it was impossible to tell if they would develop or rot.

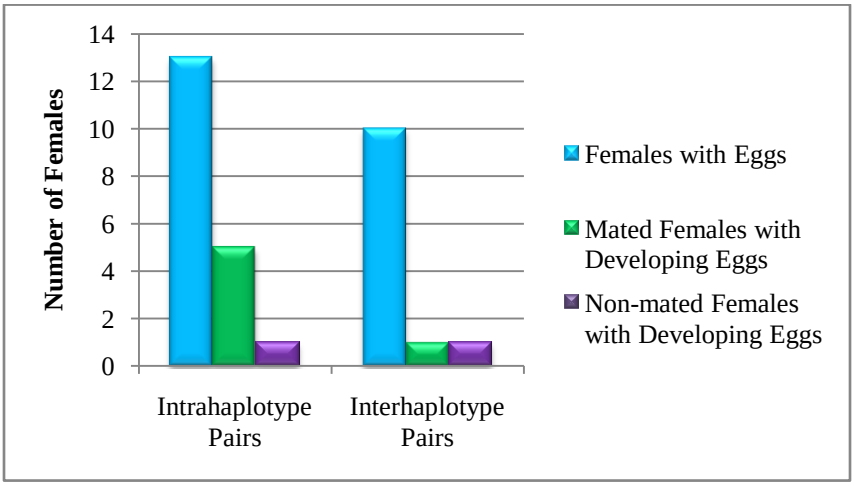

Figure 15: Females with eggs from intrahaplotype and interhaplotype pairs

Discussion

The results of this project suggest that the *O. quinebaugensis* lineage is in the process of speciation from the *O. virilis* lineage. The glaciers characteristic of the Pleistocene period may have been responsible for the initial divergence of the two populations (Mayden, 1988). These glaciers created conditions that added biogeographical barriers in areas that had been previously continuous and also acted to disperse organisms to different areas where they sometimes became isolated and diverged. These events have been hypothesized as a possible mechanism for the divergence and speciation of freshwater fish species (Mayden, 1988), so it is possible that it is also the mechanism of divergence in our two populations.

The initial divergence of the two populations most likely resulted in the initiation of an allopatric speciation between the two populations. Allopatric speciation occurs when two or more populations of the same species are isolated and diverge into separate lineages, whereas

sympatric speciation occurs when a population that is not isolated diverges (Gavrilets, 2003). The divergence of two allopatric populations usually occurs due to adaptations to differences in environmental conditions through natural selection, as well as genetic drift (random changes in allele frequencies) within each population (Wu, 2001). Some populations are isolated and begin allopatric speciation, but then continue with sympatric speciation if they are reintroduced to one another (Ayala et al., 1974). When populations are undergoing allopatric speciation, they first become genetically differentiated. If the two diverging populations come back into contact, they will either differentiate further to produce reproductive isolation or they may introgress back into one species.

The data obtained in this project suggested that the allopatric speciation process between the two populations has resulted in genetic differentiation. This is seen in the COI mtDNA, in which two distinct haplotypes were recognized, and in the allele size data for the microsatellite containing locus (Ov54). The majority of the crayfish population with the *O. virilis* haplotype had the AA Ov54 genotype while the majority of the crayfish population with the *O. quinebaugensis* haplotype had the BB Ov54 genotype. This supported the findings of Mathews et al (2008) which also found evidence for differences in mtDNA haplotypes and genotypes in the two lineages studied in this project.

Along with genetic differences, there are also significant differences in the morphology of the crayfish chela and male gonopods which further supported the process of speciation. Mating trials also supported a hypothesis of speciation: intrahaplotype mating pairs were more successful than interhaplotype mating pairs. However, since a fertile clutch was present in at least one of the females from a successful interhaplotype mating trial, and it is possible that these offspring may be viable and fertile; these populations may not be completely reproductively isolated. This is difficult to assess as some females which didn't mate had fertile clutches of eggs, making it hard to determine the paternity of the offspring of those who did. However, if the two populations are not reproductively isolated, this would suggest that the speciation process is not complete.

Although we do not know whether *O. virilis* is truly an invasive species, the difference seen in the morphology and genetics of the two populations suggests that they have only recently come into secondary contact after their divergence. If the secondary contact were older, we wouldn't expect to see a distinction between the two populations since it seems as they are

capable of introgression (Ayala et al., 1974) Now that this secondary contact has occurred, there are two distinct possibilities for the futures of the populations. The lineages may continue to diverge through sympatric speciation or they may introgress through hybridization. If the two populations create only sterile hybrids, then they will continue through sympatric speciation, however this may also occur if the two populations can produce fertile hybrids, through reinforcement. Reinforcement occurs when the hybrids of two populations have lower fitness than their parental species (Epifanio and Philipp, 2001). In this case, there would be a negative consequence associated with hybridizing, and it would therefore be selected against. The populations would evolve pre-zygotic reproductive barriers that would minimize the possibility of hybridization and serve to reinforce the speciation process by preventing introgressive hybridization (Hoskin et al., 2005). These barriers may also lead to total reproductive isolation.

The data from this project suggest that there may be reproductive barriers in place between the *O. virilis* and *O. quinebaugensis* populations as intrahaplotype mating trials were more successful than interhaplotype trials. The results of the logistic regression suggest that male morphology of the chelae and gonopods could be used as predictors of the mitochondrial haplotype. This suggests that there was a divergence of morphological characteristics between the two populations, which may either be attributed the allopatric period of their histories, or possibly to reinforcement. Since male gonopods function in reproduction, it first seemed as though the difference in gonopods may serve as a reproductive barrier if the difference interferes with the ability of the two populations to reproduce successfully. It was also thought that the difference in male chelae shape may also act in a similar way if they function in mate choice and identification. However, in an investigation by Taylor and Knouft (2006) it was found that closely related sympatric *Orconectes* are more likely to have the same or similar reproductive morphology than is expected. This suggests that male reproductive morphology may not be an important reproductive barrier in sympatric *Orconectes*. If it was an important barrier, we would have expected that sympatric *Orconectes* populations would show more divergence. Although reproductive morphology may not serve as a strong reproductive barrier, the significant difference in carapace length between the two populations may function in this way. The investigation of interpopulation mating success and carapace length difference within the pairs suggested that mating attempts and success were lowest when there was a large size difference

between the male and the female. This provides some support for size difference as a reproductive barrier; however, the sample size was too small to draw strong conclusions.

During the mating trials, we noted that the *O. virilis* intrahaplotype trials (*Ov* x *Ov*) were more successful than *O. quinebaugensis* intrahaplotype trials (*Oq* x *Oq*). One explanation for this difference may be that there was a variation in mating seasons between these two populations. Since mating trials took place in late December, it is possible that the *O. quinebaugensis* population was nearing the end of its mating season and some individuals were no longer reproductively receptive. In addition to noticing a difference in mating success, we also noted that the white glair glands of the *O. virilis* females appeared approximately two weeks before the *O. quinebaugensis* females, further supporting a possible difference in mating seasons between the two populations. If there is a difference between the mating seasons of the populations, this may act as another reproductive barrier between the two populations. Further laboratory trials or field observations of both populations would be required to determine whether these divergent morphological and mating characteristics are truly reproductive barriers between the populations.

While it is possible that reproductive barriers are reinforcing the speciation process between the two populations, it is also possible that introgressive hybridization may be working against the speciation process if there are not strong enough reproductive barriers present. Introgressive hybridization occurs when two populations are capable of producing fertile offspring which then interbreeds with one or both of its parental species (Lapedes, 1974). This introgression causes a gene flow between the two populations which lowers their genetic diversity. While the crayfish did show a preference for intrahaplotype mating, we did observe interhaplotype mating trials which resulted in developing clutches of eggs. If the interhaplotype mating trials result in fertile hybrids which can breed with their parental species, it is very possible that introgressive hybridization could occur in nature. In addition to the success of interhaplotype mating trials in the lab some of the genetic data also supported the possibility of introgressive hybridization in nature.

As previously mentioned, the genotypes seemed to have a strong correlation with mitochondrial haplotype, suggesting that they are lineage specific. However, there were a minority of cases in which individual crayfish had the mitochondrial haplotype and genotype attributed to opposing lineages. In addition, there were some crayfish in both populations with the AB genotype. These data indicate that hybridization may be occurring in nature between

these two populations. If the genotypes are truly lineage specific, it seems as though a crayfish with the AB genotype would suggest an F1 hybrid, while a crayfish with opposing mitochondrial haplotype and genotype would represent an instance of introgression.

If introgressive hybridization is occurring, this could interfere with the process of speciation that we believe is taking place with between the two species. If the *O. virilis* population is present in large numbers and hybridizes with *O. quinebaugensis*, the resulting hybrid would most likely mate with *O. quinebaugensis* crayfish, slowly introducing the *O. virilis* genes into the native population. If this gene flow is strong enough, it may lead to the local or total extinction of *O. quinebaugensis.* This possibility is consistent with our findings that individuals we collected from the R1 site were all *O. virilis* as they may have already caused local extinction of *O. quinebaugensis* in that location. Other studies have suggested that introgression may have a potential role in the extinction of freshwater species (Perry et al., 2002) and our data provides some support for this possibility.

Future Research

While we have some preliminary predictions of the relationship between *O. virilis* and *O. quinebaugensis*, there are still many unanswered questions. Further research is necessary to understand which of the mechanisms described above is currently acting on the populations. To further study whether introgressive hybridization is plausible, it would be necessary to rear any offspring of interhaplotype mating trials to determine whether they are viable and fertile. The survival rate of hybrids could be compared to that of the intrahaplotype offspring to determine whether there is any difference in relative fitness between the hybrids and intrahaplotype offspring. In addition, mating trials could be used to determine whether F1 hybrids are capable of mating with individuals from either of the parental populations, and whether these trials would result in offspring.

To further investigate the possibility of reinforcement, trials could be used to determine whether females are selective in their mating choice. Since the females were not given the choice of two males in our mating trials, they may have only mated due to lack of choice. A series of trials could be used to determine whether females would choose a male of the same lineage over a male of the opposite lineage. In addition, it would be beneficial to study other male and female characteristics which may play a role in mate choice to determine whether there is a difference

between the two populations, as this would provide further insight into the possibility of reinforcement.

Work Cited

Abernathy, K. (1994) The establishment of a hybrid zone between red and sika deer (genus *Cervus*). *Molecular Ecology* 3: 551-562

Anttila, C. K., Daehler, C. C., Rank, N. E. and Strong, D. R. (1998) Greater male fitness of a rare invader (*Spartina alterniflora*, Poaceae) threatens a common native (*Spartina foliosa*) with hybridization. *American Journal of Botany* 85: 1597-1601

Avise, J. (2004) Molecular Markers, Natural History, and Evolution, 2nd ed. Sunderland, MA: Sinauer Associates, Inc.

Ayala, F., Tracey, M., Hedgecock, D. and Richmond, R. (1974) Genetic differentiation during the speciation process in drosophila. *Evolution 28*: 576-592

Beebee, T. and Rowe, G. (2008) An Introduction to Molecular Ecology, 2nd ed. New York: Oxford University Press.

Bergey, E. A., Jones, S. N. and Fenolio, D. B. (2004) Annual report: surveys and studies of Oklahoma crayfish and the Grotto salamander. Oklahoma Biological Survey: The University of Oklahoma.

Berrill, M. (1985) Laboratory induced hybridization of two crayfish species, *Orconectes rusticus* and *O. propinquus*. *Journal of Crustacean Biology* 5:347-349

Butler, M. J. and Stein, R. A. (1985) An analysis of the mechanisms governing species replacements in crayfish. *Oecologia* 66:168-177

Capelli, G. M. and Capelli, J. F. (1980) Hybridization between crayfish of the genus *Orconectes*: Morphological evidence (Decapoda, Cambaridae). Crustaceana 39:121-132

Crocker, D. W. (1979) The Crayfishes of New England. *Proceedings of the Biological Society of Washington 92*: 225-252

Crocker, D. W. and Barr, D. W. (1968) Handbook of the crayfishes of Ontario. University of Toronto Press, Toronto

de Queiroz, Kevin. (1998) The General Lineage Concept of Species, Species Criteria, and the Process of Speciation. *Endless Forms: Species and Speciation*. Print.

DiDonato, G. W. and Lodge, D. M. (1993) Species replacements among *Orconectes* crayfishes in Wisconsin lakes: The role of predation by fish. *Canadian Journal of Fisheries and Aquatic Sciences* 50:1484-1488

Epifanio, J. and Phillip, D. (2001) Simulating the extinction of parental lineages from introgressive hybridization: the effects of fitness, initial proportions of parental taxa, and mate choice. *Reviews in Fish Biology and Fisheries 10*: 339-354

Fetzner, J. (2008) State of Massachusetts- Crayfish Species Checklist. http://iz.carnegiemnh.org/crayfish/country_pages/state_pages/massachusetts.htm

Garvey, J. E. and Stein, R. A. (1993) Evaluating how chela size influences the invasion potential of an introduced crayfish (*Orconectes rusticus*). *The American Midland Naturalist* 129:172-181.

Garvey, J. E., Stein, R. A. and Thomas, H. T. (1994) Assessing how fish predation and interspecific prey competition influence a crayfish assemblage. *Ecology* 75:532-547

Gavrilets, S. (2003) Perspective: models of speciation: what have we learned in 40 years? *Evolution 57*: 2197-2215

Gilbert, F. (2004) *Developmental Biology*, 8th ed. Sunderland, MA: Sinauer Associates, Inc.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Hardig, T., Brunsfeld, S., Fritz, R., Morgan, M. and Orians, C. (2000) Morphological and molecular evidence for hybridization and introgression in a willow (*Salix*) hybrid zone. *Molecular Ecology 9*: 9-24

Hebert, P., Cywinska, A., Ball, S. and deWaard, J. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society 270*: 313-321

Hill, A. M., Lodge, D. M. (1999) Replacement of resident crayfishes by an exotic crayfish: the roles of competition and predation. *Ecological Applications 9*: 678-690

Hill, A. M., Sinars, D. M. and Lodge, D. M. (1993) Invasion of an occupied niche by the crayfish *Orconectes rusticus*: Potential importance of growth and mortality. *Oecologia* 94:303-306

Hoskin, C., Higgie, M., McDonald, K. and Moritz C. (2005) Reinforcement drives rapid allopatric speciation. *Nature 437*: 1353-1356

Keller, R., Frang, K., and Lodge, D. (2008) Preventing the spread of invasive species: Economic benefits of intervention guided by ecological predictions. *Conservation Biology 22*: 80-88

Lapedes, Daniel N. Editor in Chief (1974) McGraw-Hill Dictionary of the Life Sciences. New York: McGraw-Hill Book Company.

Lodge, D. M., Kratz, T. K. and Capelli, G. M. (1986) Long-term dynamics of three crayfish species in Trout Lake, Wisconsin (USA). *Canadian Journal of Fisheries and Aquatic Sciences* 43:993-998

Lodge, D. M, Taylor, C. A, Holdich, D. M and Skurdal, J. (2000) Nonindigenous crayfishes threaten North American freshwater biodiversity: Lessons from Europe. *Fisheries 25*: 7-20

Mathews, L., Adams, A., Anderson, E., Basile, M., Gottardi, E. and Buckholt, M. (2008) Genetic and morphological evidence for substantial hidden biodiversity in freshwater crayfish species complex. *Molecular Phylogenetics and Evolution 48*: 126-135

Mathews, L and Warren, A. (2008) A new crayfish of the genus *Orconectes* Cope, 1872 from southern New England (Crustacea: Decapoda: Cambariidae). *Proceedings of the Biological Society of Washington 121*: 374-381

Mayden, R. (1988) Vicariance biogeography, parsimony, and evolution in North American freshwater fishes. *Systematic Zoology 37*: 329-355

Mayr, Ernst. (1996) What Is a Species, and What Is Not? *Philosophy of Science* 63: 262-277

Mooney, H. and Cleland, E. (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Science of the United States of America 98*: 5446-5451

Mullis, K., Erlich, H., Arnheim, N., Horn, G., Saiki, R. and Scharf, S. (1986) Process for amplifying, detecting, and/or cloning nucleic acid sequences. US Patent 4,800,159, filed December 17, 1986, and issued January 24, 1989.

Noor, M. A. F., Grams, K. L., Bertucci, L. A. and Relland, J. (2001) Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences 98-21*: 12064-12068

Nucleics (2009) Exonuclease I - Shrimp Alkaline Phosphatase clean up of PCR products. http://www.nucleics.com/DNA_sequencing_support/exonucleaseI-SAP-PCR-protocol.html

Perry, W. L., Feder, J. L. and Lodge, D. M. (2001a) Hybrid zone dynamics and species replacement between *Orconectes* crayfishes in a northern Wisconsin lake. *Evolution* 55:1153-1166.

Perry, W. L., Feder, J. L. and Lodge, D. M. (2001b) Implications of hybridization between introduced and resident *Orconectes* crayfishes. *Conservation Biology* 15:1-11.

Perry, W. L., Lodge, D. M. and Feder, J. L. (2002) Importance of Hybridization Between Indigenous and Nonindigenous Freshwater Species: An Overlooked Threat to North American Biodiversity. *Systematic Biology 51*: 255-275

Purves, William K. et al (1995). *Life: The Science of Biology, Fourth Edition*. Sunderland, MA: Sinauer Associates Inc.

Rees, D. J., Dioli, M. and Kirkendall, L. R. (2002) Molecules and morphology: evidence for cryptic hybridization. *Molecular Phylogenetics and Evolution 27*: 131-142.

Reynolds, J.D. (2002) Growth and Reproduction. *Biology of freshwater crayfish.* Nottingham, UK: University of Nottingham.

Rhymer, J. and Simberloff, D. (1996) Extinction by hybridization and introgression. *Annual Review of Ecological Systems 27*: 27-86

Rohlf, F. J. (2005) tpsDig, digitize landmarks and outlines, version 2.05. Department of Ecology and Evolution, State University of New York at Stony Brook.

Sanger, F, Nicklen, S and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the Natural Academy of Sciences of the United States 74*: 5463-5467

Schwarz, D., Matta, B., Shakir-Botteri, N. and McPheron, B. (2005) Host shift to an invasive plant triggers rapid animal hybrid speciation. *Nature 43*: 546-549

Smith, D. G. (1975) Variability of crayfish of the virilis section (Cambaridae: *Orconectes*) introduced into New England and eastern New York. *The American Naturalist 102*:388-391

Smith, D. G. (1981) Evidence for hybridization between two crayfish species (Decapoda: Cambaridae: *Orconectes*) with a comment on the phenomenon in Cambarid Crayfish. *The American Midland Naturalist* 105:405- 407

Stevens, G., North, B. (1974) *Biology*. New York, NY: John Wiley & Sons Inc.

Taylor, C. and Knouft, J. (2006) Historical influences on genital morphology among sympatric species: gonopod evolution and reproductive isolation in the crayfish genes *Orconectes* (Cambaridae). *Biological Journal of the Linnean Society 89*: 1-12

Tierney, A. J. (1982) Individual variation and taxonomic significance of the seminal receptacle of the crayfish, *Orconectes propinquus* (Girard) (Decapoda, Cambaridae). *Crustaceana* 43:284-296

United States Environmental Protection Agency (2009) Crayfish Species. http:// yosemite.epa.gov/ r10/ ECOCOMM.NSF/Invasive+Species/Crayfish

United States Geological Survey (2009) http://nas.er.usgs.gov/taxgroup/Crustaceans/ maps/or_virilis.gif

Voet, D., Voet, J. and Pratt, C. (2008). *Fundamentals of Biochemistry*, 3rd ed. Hoboken, NJ: John Wiley & Sons, Inc.

Wilson, K. A., Magnuson, J. M., Lodge, D. M., Hill, A. M., Kratz, T. K, Perry, W. L. and Willis, T. V. (2004) A long-term rusty crayfish (*Orconectes rusticus*) invasion: dispersal patterns and community change in a north temperate lake. *Canadian Journal of Fish and Aquatic Science 61*: 2255-2266

Wu, C. (2001) The genic view of the process of speciation. *Journal of Evolutionary Biology 14:* 851-865